IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

Applicants:	D.R. Meldrum et al.	Attorney Docket No.:	UWOTL126696
Application No.:	10/698,234	Art Unit:	1743 / Confirmation No: 2313
Filed:	October 30, 2003	Examiner:	N.A. Levkovich
Title:	PREPARATION OF SAMPLES AND SAMPLE EVALUATION		

DECLARATION OF INVENTORS UNDER 37 C.F.R. § 1.131

Seattle, Washington 98101

June 13, 2006

TO THE COMMISSIONER FOR PATENTS:

The undersigned declare as follows:

1. I am a named co-inventor in the above-referenced patent application (the "Present Application");

2. At least as early as May, 2001, I participated in the preparation of a research plan (the "Research Plan") directed to developing underlying technology, protocols and designs for fast, automatic crystal preparation, growth, optimization, cryocooling, and mounting of biomacromolecular crystals for X-ray crystallography, the Principal Investigator of which is Dr. Deirdre R. Meldrum.

3. A true and correct copy of relevant portions of the Research Plan is attached hereto as Exhibit A. On information and belief, the revision date of "05/01" printed in the footer of the Research Plan accurately indicates that this revision of the Research Plan was completed no later than May, 2001.

4. On information and belief, the Research Plan has never been published or otherwise publicly disclosed.

5. The Research Plan discloses an apparatus and a method of preparing and handling protein samples for crystallography studies of protein crystals, that uses capillary tubes that are

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open at the ends, and dispensing the fluid into the capillary in several bursts of about 400 droplets, alternately firing the droplets and sucking the fluid into the capillary, then closing the ends of the tube. A crystallographic analysis of the fluid is subsequently conducted *in situ*.

6. In my opinion, the Research Plan discloses the invention claimed in the Present Application in sufficient detail to enable a person of ordinary skill in the relevant art to practice the invention claimed in the Present Application, without undue experimentation.

7. The Research Plan was prepared to assess interest in, and to obtain funding for, research related to protein crystallography. The invention disclosure was then diligently disclosed to the University of Washington's Technology Transfer office, which subsequently submitted it to an outside patent counsel for preparation and filing.

I hereby declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true, and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under § 1001 of Title 18 of the United States Code, and that such willful false statements may jeopardize the validity of the application or any patent issued thereon.

Executed at Seattle (city), Washington (state), this 26th day of June, 2006.

Deirdre R Meldrum

Deirdre R. Meldrum

Executed at _____ (city), _____ (state), this _____ day of _____, 2006.

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Wim G. J. Hol

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Research Plan

A. Specific Aims

Crystal growth is an essential step in obtaining three-dimensional structural information about biomacromolecules such proteins by X-ray diffraction. The proposed Research Plan will develop comprehensive methods, technology, instrument designs, and fully operational hardware for high throughput, automated preparation, growth and analysis of protein crystal samples in a capillary format.

The ultimate aim of the project is to develop the technological framework, and then an operational prototype, of an Integrated Protein Crystallization System. This system will provide high-throughput, fully automated sample processing in a single unbroken pipeline that extends from incoming purified proteins to outgoing crystal samples ready for crystallographic analysis.

Broad Aim

We aim to develop underlying technology, protocols, and designs for fast, automatic crystal preparation, growth, optimization, cryocooling, and mounting of biomacromolecular crystals for X-ray crytallography. Based on this research, we further aim to **build and test a prototype of an Integrated Protein Crystallization System** that will automatically handle the entire crystallographic sample process, from purified protein to crystallographic analysis, with a throughput of greater than 500 samples/hour.

Specific Aims

1. Develop methods for handling very small protein sample volumes (50 nanoliters and smaller).
2. Develop and optimize protocols for crystal growth in a capillary environment, under a variety of diffusion conditions (gas and liquid).
3 Develop an automated growth and storage environment for capillary-based samples, to allow automated evaluation of crystal growth *vs* sample conditions, building on estabished electronics-industry handlings techniques such as "tape and reel" transport.
4. Develop an automated co-crystallant Piezo-dispenser hotel capability, allowing 100 or more reagents to be automatically used in sample preparation for determination of optimum crystallization conditions.
5. Design, build and test a capillary imaging system which will allow rapid and robust automated acquisition of image data for subsequent evaluation of crystal growth.
6. Create software which automates the evaluation of the capillary crystallization experiments, via automatic detection of crystal growth and intelligent feedback to the initial sample preparation conditions.
7. Develop procedures for automatic cryocooling of crystals grown in capillaries, including directly cryoable solutions.
8. Discover, test and implement precipitating solutions which are suitable for cryocooling of crystals without the need for adding cryopreservatives.
9. Design and build locating methods, holders, and grippers which allow automatic placement of capillaries onto goniometer heads without human intervention. Create systems for efficient transfer of capillaries to X-ray analysis. Develop software which allows automatic mounting and centering of frozen crystals using images of the cryocooled crystals in the capillaries.
10. Develop a 2nd generation piezo-dispenser hotel, allowing 1000 or more reagents to be screened for the purpose of medicinal macromolecular crystallography projects.
11. Build a complete integrated processing system.
12. Test the complete Processing System in Dr. Wim Hol's Biomolecular Structures Center.

B. Background and Significance

B.1 Justification

Preparation of well-diffracting crystals is the key challenge in biomacromolecular crystallography. It is of critical importance for high-throughput structure determination for structural genomics and medicinal protein

crystallography projects.

The first challenge in crytal structure determination is the discovery of optimum crystal growth conditions. This process typically requires preparation and evaluation of thousands of samples under varying conditions. Automation of this process to increase throughput and reduce labor requirements is of enormous value to macromolecular crystallography. Once robust crystals are obtained, the next challenge is to freeze these samples to 100K without degradation of diffraction efficiency or formation of ice crystals, in preparation for X-ray data collection. The final critical step is to place the frozen crystal in the X-ray beam and center it precisely at the position where cryostream, X-rays and rotation axes of the goniometer intersect.

The aim of our research is to obtain a completely automated, integrated capability encompassing *all* of these steps. Our goal is elimination of manual intervention for crystal growth, cryofreezing and crystal location for crystallographic analysis. Based on our preliminary results, we believe that this goal is within reach. The proposed research, if successful, will significantly or even dramatically reduce the bottlenecks between protein production and the initiation of X-ray data collection for biomacromolecular crystallography, while working with initial protein volumes of 10–50 nanoliters.

Several factors give us confidence in the feasibility of this revolutionary system concept:

1. the capability of our existing ACAPELLA-5K sample preparation system (1 R24 HG02215, Meldrum), which has demonstrated the ability to prepare microliter capillary-based samples for Genomic Sequencing at a rate of 650/hour by means of piezoelectric dispensers;
2. the demonstrated ability of piezoelectric dispensers to deliver subnanoliter fluid volumes of proteins;
3. extensive past demonstration of free-interface diffusion for protein crystal growth in a capillary format, using manual sample preparation protocols [38];
4. recent demonstration that the results of (3) can be substantially duplicated when sample capillaries were filled by the ACAPELLA-5K;
5. the relative ease of automated handling of capillaries containing crystals, for transport, storage, and imaging, as demonstrated by the ACAPELLA development project;
6. the proven capability to freeze crystals inside capillaries for X-ray diffraction experiments *without* having to touch or manipulate the crystal [39,40];
7. the relative ease of 3-D location of crystals inside frozen sample capillaries, by using images from multiple directions and depths within the sample;
8. the ability to control and track capillary geometry, allowing the direct transfer of sample crystal location data to the goniometer control system of X-ray data collection instruments.

Our ACAPELLA system was not designed with protein crystal growth in mind. However, we have already shown that ACAPELLA technology offers amazingly appropriate dimensions, processing flexibility and speed for actual protein crystal growth. The proposed work will augment ACAPELLA with new capabilities for a small online co-crystallant reagent library, and an extensive small-molecule medicinal library for future use, as well as completing the rest of a processing pipeline that connects the modified ACAPELLA directly to the synchrotron. We must develop a new capillary mass storage and growth system, which must be tightly integrated with fast, robust crystal imaging hardware and software. An in-capillary-crystal freezing subsystem will be developed as well as the hardware and software needed to transfer both sample and crystal position data to the X-ray facility.

This work offers a major impact: full automation and optimization of all steps between purified protein production and X-ray data collection for structural genomics. In addition, future coupling with combinatorial libraries of chemical compounds will allow high-throughput testing for crystal growth of a target protein in the presence of thousands of different drug candidates. Clearly, both structural genomics and structure-based drug design will benefit in major ways from the technology, hardware and software this grant proposes to create.

B.2. The Importance of Macromolecular Crystallization

The basic principles of macromolecular crystallization are well understood [1-4]. Aqueous solutions of proteins are brought to a state of supersaturation, nucleation ensues, followed by crystal growth. Solubility is affected by temperature, pressure, pH, and the presence, absence, and concentrations of co-solutes [2-9]. Nucleation

is less well understood. Crystal growth is affected by as many as twenty variables [3,4]. In a high-throughput (HTP) setting the operational variables at our disposal are the concentration of the protein; the nature, pH and concentration of the buffer; the nature and concentration of the crystallizing agent; the nature and concentration of any additive present; the temperature and the method by which crystallization is affected. We will approach crystallization in two stages, namely a screening stage and an optimization stage. In the screening stage we will identify the optimal buffer, crystallizing agent, and additives that lead to crystalline outcomes. In the optimization stage we will determine which method of crystallization leads to the best crystals, and vary solute concentrations in the crystallization column bring about a successful outcome.

To bring crystallization to high throughput we need to execute and analyze large numbers of crystallization experiments, and we need to maximize the probabilities that these experiments will lead to successful outcomes. We will maximize the success rate of these experiments by the deployment of a library of co-crystallizing agents that have the potential to stabilize proteins and aid in crystallization.

B.3 Existing methods for protein crystal growth

Successful crystal growth methods must cause the protein to become supersaturated so that crystal nuclei form and then grow into crystals. Typical dimensions are 50 μm in all directions although thinner plates and needles are occasionally also successfully used with modern well-focused synchrotron beams. The most important procedures available for protein crystal growth can roughly be divided into the following techniques:

Vapor diffusion by either the hanging or sitting drop methodology

This very popular method results in a well-defined end point when the thermodynamic potential of the water in the drop is equal to that in the so-called "well solution". The progress of crystal formation is inspected over time by eye or, more recently, by photographic imaging techniques. Many reviews and books have been devoted to this procedure [2,11]. Several robotic systems are described and commercially available to set up vapor diffusion experiments– including Gilson/Cyberlab, TECAN, CARTESIAN, ORYX6, and others.

Dialysis

The protein solution is separated from a solution containing the precipitant by a thin membrane which prevents escape of the protein form the crystal growth reservoir. Micro-dialysis techniques have been introduced and have the advantage that the same protein solution can eqiuilibrate with a wide variety of precipitant solutions. The complexity of setting up the experiments, the fragility of the membrane and the difficulty in harvesting crystals make this procedure difficult for high-throughput methods.

Micro-batch under oil

This method has recently attracted considerable attention since the group of Dr. DeTitta in Buffalo has been successful in rapid automation of this procedure [12]. Pioneering work has been carried out at Imperial College London [13] and a commercially available system, the Oryx 6 from Douglas Instruments, Oxford, automates this procedure.

Liquid-liquid diffusion, or capillary crystallization

This procedure has been used for many decades and has been highly successful in the Groningen and Seattle laboratory of Wim Hol. The procedure allows for slow mixing of solutions, can use multiple layers of solutions, while storage can be very compact. The disadvantages so far are that setting capillary experiments are set up manually, and inspection of the capillaries is time consuming. This approach is the basis for the proposed Integrated Crystallography Processing System. Other procedures are essentially variants of the above major techniques. Some variants use gel formation to slow down diffusion rates, or employ the diffusion of volatile buffers through the vapor phase.

B.4 Crystal Growth Screens, Follow-ups and Precipitating Solutions

Strategies and Solutions for Protein Crystal Growth

Several different strategies have been proposed over the years for probing crystallization space. Systematic screens, where a small number of variables are changed in small steps, are often used and provide a detailed picture of the effect of a small number of variables on precipitation or crystallization properties of the target

protein. Often this procedure is used in follow-up and optimization steps. For initial screening the random matrices as pioneered by Jancarik and Kim [16] have become very popular. Hampton, Emerald Biostructures and other companies sell such screens. Sophisticated mathematical approaches have also been proposed to optimally design and analyze screens [17].

The precipitating agents used for inducing nucleation and crystal growth are under continuous development [19,20] but the major precipitating agents have so far been salts, organics and polymers, in particular PEGs. Combinations of these are often used as well such as PEG-salt combinations. Several books and monographs have been published that describe the precipitating agents in use. Organic buffers are quite common since they may prevent the formation of undesired salt crystals, but many proteins have also crystallized out of phosphate buffers, for example. From the very early days of protein crystallography it has been clear that small molecule additives can have a dramatic effect on crystal growth such as in the case of the addition of dioxane to remove the twinning in the early studies on chymotrypsin [21]. More recently, additive screens have been developed to explore more systematically evaluate the effect of certain additives [22]. The laboratory of Wim Hol has quite a few examples of the positive effect of small molecules on the formation of crystal growth. These include inhibitors and substrates which were essential to obtain any crystals of Mycobacterium tuberculosis Dihydrofolate reductase [23], M tuberculosis DHPS [24], L' mexicana and others[25,26]. The additive binds at the active site and somehow, most likely by decreasing the mobility of the protein molecules, promotes crystal formation. Other compounds are not binding in active sites but settle between molecules in the crystal lattice, Examples are the MDT in cholera toxin which was engaged in numerous crystal contacts [27] This has been followed up by the ongoing synthesis of "co-crystallants" within the context of the SGPP (Structural Genomics of Pathogenic Protozoa) consortium, where the effect of co-crystallants will be systematically tested [28].

B.5 Current Methods for Cryocooling, Mounting, and Positioning of Crystals

Cryocooling

The various crystallization solutions fall into two distinct categories: those that can be frozen directly without formation of ice crystals, and those that need the addition of a "cryo-protectant" to make safe freezing of a protein crystal possible. "Directly Cryoable (DC) Solutions" are obviously highly desirable since in many instances these allow mounting and freezing of crystals without having to prepare and search for the proper cryoprotectant. Often crystals are very sensitive to handling even without changing mother liquor at all – and hence procedures which minimize crystal handling have numerous advantages. Our capillary-based methods will allow for addition of cryoprotectant at later stages, but the major thrust will be to grow crystals out of DC Solutions. Hence we have begun to check the DC properties of various solutions, as well as of new crystallization mixtures. We will continue our search for DC solutions as part of the proposed project.

Mounting

By far the most popular mounting method is to use a small loop to scoop the crystal out of a solution [29], The sample is then flash frozen in place, thereby combining the cooling and mounting steps.

For loop mounting, the solution can either be the original mother liquor or, quite often, when this mother liquor leads to water crystal formation during the cooling process, an artificial mother liquor with cryoprotectant added, such as glycerol, sugars or other compounds. Mounting with certain oils can also prevent undesired ice formation in the protein crystal during cryocooling.

The feasibility of direct cryocooling of crystals in capillaries has been shown by the group at Granada, as well as in our own preliminary experiments (Section C.5).

Crystallograhic Interface and Positioning

Until recently, cryocooled crystals in loops or on pins were placed by hand onto the goniometer of the diffraction pattern recording device – whether in-house or at the synchrotron. Recently, several robotics systems have been successfully developed to place frozen crystals onto area detectors for data collection [30,31]

B.6 The State of Crystallographic Automation

Until quite recently almost all crystallization experiments were performed by hand. A few early automated systems used standard robotic liquid handling systems, such as one developed in the Hol Lab in Groningen

[32]. Only in the last five to ten years have a significant number of automated systems been developed specifically for protein crystallography. See (Stevens 2000) for a review of high-throughput protein crystallization and (Abola et al. 2000) for an overview of automation of X-ray crystallography [33, 34]. Most if not all of the automated systems mentioned below do **not** automate the entire process from purified proteins as an input to crystal growth and identification of crystals to providing frozen and mounted crystals for data collection. As stated by Chayen et. al., optimization for crystal growth must be improved, automated and adapted for high-throughput to remove the main bottleneck in structural genomics [35].

Currently available commercial robotic systems for protein crystallography include the Gilson/Cyberlab (Middleton, WI) C-200 PC Workstation (www.gilson.com) that prepares up to six 24-well plates with up to 4 proteins and dispense volumes of 1 uL or more for standard hanging drop and sitting drop vapor diffusion crystallography; the Douglas Instruments Ltd. (Hungerford, Berkshire, U.K.) Oryx 6 [and IMPAX 1-5] (www.douglas.co.uk/oryx.htm) for microbatch or sitting/hanging drop vapor diffusion methods, utilizing 0.2 to 1 μL per crystallization (2 μL typically) for up to 72 crystallization trials/run. Cartesian Technologies, Inc. (Irvine, CA) has a ProSys 96 (www.cartesiantech.com/crystallography.html) instrument with 96 synQUAD pumps for hanging drop and sitting drop experiments utilizing up to 96 different reagents with a minimal aspiration volume of 1 μL. TomTec (Hamden, CT) utilizes their existing Quadra-Plus system (www.tomtec.com/Pages/Uniwell.html) with 24 of their new Uniwell strips in a Uniwell frame for sitting drop crystallography experiments. The Uniwell storage system houses the proteins and facilitates storage.

There are companies developing **in-house** capabilities for high-throughput protein crystallography such as SYRRX (San Diego), Structural GenomiX (SGX) (San Diego, CA), Altus (Cambridge, MA), Bio-Xtal (Gif sur Yvette, France), BSI Proteomics (Gaithersburg, MD), GeneFormatics (San Diego, CA), CrystalGenomics (Taejon, Korea), Fluidigm Corp. (South San Francisco, CA), and Affinium Pharmaceuticals (Toronto, Ontario, Canada). SYRRX (www.syrrx.com) has a robot called Agincourt that is based upon technology presented in (Santarsiero et al. 2002, Stevens 2000) [33.36]. This robot is used to typically prepare total drop volumes of 100 nL (50 nL protein plus 50 nL reservoir solution) in hanging-drop or sitting-drop vapor-diffusion configurations to produce crystals that can be directly used for X-ray data collection. The system is capable of volumes as small as 20 nL. Smaller volumes promote faster vapor-diffusion equilibration between drops, result in reduced crystal growth times and are more easily cryocooled. It is believed that the current instrument can deliver 30,000 drops per hour making it the fastest system. Structural GenomiX (www.stromix.com) utilizes some form of proprietary robotic technology for automated crystallization screens. In January 2001, they formed a collaboration with Caliper Technologies Corp. (Mountain View, CA) for joint development of a LabChip-based protein analysis system (www.stromix.com/news/pr_010801.html) but no further information is available. BSI Proteomics (www.bsiproteomics.com) has developed an Automated Robotics Dynamically Controlled Crystallization System (DCCS) (ARD) that maintains dynamic control of the entire equilibration process and screens 100 independent precipitant conditions in parallel. They utilize dialysis-based crystallization with a variable volume requirement (2 μL to 40 μL). They are currently working on technology to utilize magnetic levitation to provide the benefits observed on protein crystal growth in space. Again, these systems are **not** available for external use.

Fluidigm (www.fluidigm.com) has a new Topaz Starter Kit Prototype for protein crystallography that uses a disposable Multi-Layer Soft Lithography (MSL) chip (2 inches x 3 inches) to screen 48 reagents per chip. Protein consumption is less than 20 nL and total protein consumption is less than 5 μL for 144 conditions tested per chip (48 screening reagents x 3 ratios). This is a prototype device. Although it is not clear what performance is achieved or what manual labor is required to prepare and operate the Topaz system, it is most likely the lowest volume system available. It is also not clear how the crystals are accessed for imaging and further analysis.

Several institutions have developed their own high-throughput protein crystallography facilities. One of note is George DeTitta's system at the Hauptman-Woodward Research Institute (Buffalo, NY) in collaboration with Queen's University and the University of Toronto [12]. Their system is capable of performing 40,000 microbatch experiments per day using 1536-well Greiner microplates.

Crystal Growth in Capillaries

Protein crystals have long been grown in capillaries. Advantages of capillaries include small volumes, no drop dispersion as in hanging drop methods, potential for controlled evaporation/diffusion via small exposed surface

area at the ends of the capillaries, suitability for small-molecule samples, and no crystal manipulation [37]. Capillaries enable free interface diffusion, resulting in diffraction quality crystals, as shown by numerous examples from the Hol group [38].

A Spanish group has been very active in crystallographic structure determination from protein to electron density inside glass capillaries without crystal handling [39,40]. Crystals are grown in cryobuffer (20% glycerol) in X-ray capillaries, a laser pointer and a nitrogen stream in a trap flash-cool the crystals, and X-ray diffraction analysis is performed. Crystal cracking or dissolution is minimized since the crystals remain in the capillary throughout the entire process.

Automated Mounting and Indexing of Samples

A conceptual design from the Stanford Synchrotron Radiation Laboratory (SSRL) for automated macromolecular crystal sample storage, mounting and characterization is presented by Abola et al [34]. They show a 96-pin cassette that holds 96 crystals at 77°K, and a robotic system that extracts individual samples from the storage device and mounts them on a diffractometer at the synchrotron beam line. The cassette is bar-coded and each crystal location is identifiable by a coordinate system, allowing for unique identification of each sample.

The bioinstrumentation group at Lawrence Berkeley National Lab (LBNL), in collaboration with Advanced Light Source (ALS), the Genomics Institute of the Novartis Research Foundation (GNF), and SYRRX, Inc. are reported to be developing an automated crystal recognition and alignment system for synchrotron beam lines [34]. A standard cryovial is moved to a goniometer. An electromagnet holds the magnetic cap and sample while the vial is replaced into dewar. Crystal alignment is semi-automated with a software/manual hybrid.

A method for mounting capillary tubes for cryogenic macromolecular crystallography is presented by Nakasako et al. [41]. The purpose is to eliminate the disadvantages of a free-standing thin film (wire loop): the blind region where incident or diffracted X-rays hit the loop and the difficulty of aligning crystals. A conventional capillary tube of 10 μm thickness is used so it does not prevent the transmission of X-rays. Crystals can be aligned utilizing the rigid wall of the capillary tube. The capillary is sliced with a dicing machine to make a ring that is glued to a glass fiber, placed on a brass pin with a magnet, and positioned in the cooled nitrogen gas stream.

Automated Crystal Imaging

A variety of systems for in-situ imaging for detecting crystallization have been developed. Most are designed for planar geometries associated with "hanging drop" or "sitting drop" methods. Such systems have been implemented at Buffalo, and are under development at companies such as SYRRX. TECAN is developing high-throughput imaging systems which allow scanning, as is TRITEK. We are not aware of any system which has been developed for high-throughput imaging of in-capillary crystal growth. Development of such a subsystem is a major aim of the proposed project.

Automated Image Analysis and Detection

The detection of the progress of crystal growth in wells or drops by automated methods is the subject of intense research – both in academia and industry. It is hard to obtain a precise picture of the quality of the various software packages, but few if any groups have reported that they have solved the crystallization detection problem. Active research is going on in the labs of DeTitta, of Victor Lamzin, and also at SYRRX, and probably in many other places. The detection of clear drops as well as of very well defined and sizeable crystals is not the major difficulty, The real challenges are the detection and categorizing of the various forms of precipitates which can range from milky clouds, sandy grain, ultra-thin stacked plates, clusters of thin needles, angel hair, and various other shapes and forms.

B.7 Medicinal Macromolecular Crystallography

Macromolecular crystallography provides deep insight into the folds of proteins, DNA, protein-DNA complexes, membrane proteins, the ribosome and most recently even membrane-enveloped viruses [42]. In this way macromolecular crystallography has contributed enormous insight into cellular processes at the atomic level. Macromolecular crystallography also can contribute to the development of new medicinal drugs via the structure-based drug design cycle [43,44]. Several major new drugs, including HIV protease inhibitors and anti-influenza neuraminidase drugs, have been based on, often numerous, crystal structures of complexes of candidate

drugs with the target protein. It is likely that the future will see the power of automation being used for bringing together hundreds if not thousands of compounds from chemical compound libraries with potential drug target proteins and other biomacromolecules. This will require that future robots not only be able to handle numerous different proteins, but also to set up co-crystallization experiments of a single macromolecular target, or a series of variants of such a target, with compound libraries. The proposed capillary crystal growth robot will handle the set-up of numerous co-crystallization experiments, paving the way for unraveling structures of drug target proteins in complexes with small molecules at a larger scale than ever before.

C. Preliminary Studies

C.1 Status and Capabilities of the Acapella-5K

The proposed Research grows directly out of our ACAPELLA-5K Automated Liquid Sample Processor Advanced Development at the Genomation Laboratory of the University of Washington [45–47]. The heart of past and current GNL research is automated, *integrated* sample processing systems handling nanoliters to microliters of fluid (NIH NHGRI 1 R01 HG01497, 1 R24 HG02215). The essence of ACAPELLA is fluid handling in capillaries (Figure C1). The capillary provides a single sample carrier that is inexpensive, small in volume, clean, and well-suited to robotic handling. For Genetic Sequencing applications, ACAPELLA has been optimized for sample volumes in the range of 0.5-2 μl. There is no fundamental limitation in handling substantially smaller volumes. The second-generation Acapella-5K system shown in Figure C.2 (and also in the video in the Appendix) handles 5,000 reactions in 8 hours.

Architecture

Figure C.3 shows the ACAPELLA-5K processing architecture. ACAPELLA is a modular system, so that it can be readily adapted to other applications. At present, two modules are fully developed: (i) the Sample Preparation or "Core Processor" module, followed by (ii) the Thermal Cycling module [48]. Only the ACAPELLA Core Processor, represented in green in Figure C.3, is part of the concept presented in this proposal.

Capillary Dispenser

The process begins with dispensing of empty glass capillaries [50]. Batches of capillaries, which are never reused, are loaded into a capillary "hopper", which stores about 5000 capillaries and dispenses them, one at a time on command, into the Core Processor.

Rotor and Capillary chucks

The A5K Core Processor operates in a circular pipeline. The heart of the processor is a rotating platform that carries 18 identical capillary handling chucks (Figure C.4). Each chuck retains one capillary via a special purpuse seal. The chuck also includes an integral miniature piezoelectric diaphragm pump to allow movement of the fluid column within the capillary. The rotating platform includes the electronics necessary to drive the mixing function of the chucks.

Empty capillaries are loaded onto the rotor by insertion into a chuck. They travel counterclockwise through the various processing steps, and then are offloaded from the rotor again at the lower left after almost a full revolution of motion. The rotation rate of the rotor is approximately 1 revolution per 90 seconds.

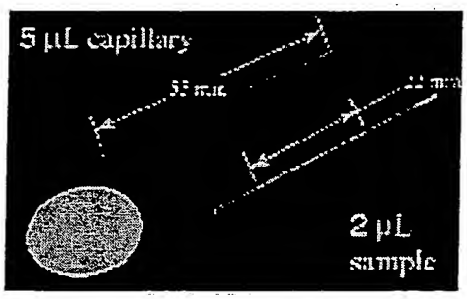

Figure C.1. 5 µl capillary format (for 0.5-2 µl sample volumes) for high-throughput operations in ACAPELLA. Typical capillary dimensions are 55 mm length, 0.8 mm outer diameter, and 0.3 mm inner diameter..

Aspiration Stage

Incoming DNA samples from a microplate are first added to the capillary by aspiration. The Core Processor aspirates DNA from a designated plate well into each empty capillary. The aspiration step is performed by turning the respective chuck and empty capillary downward, and dipping into the plate.

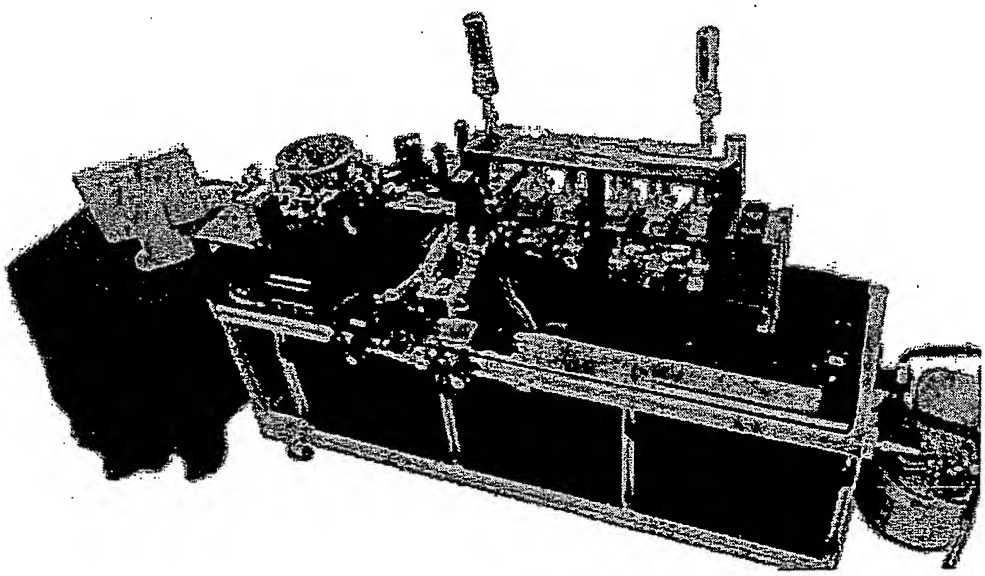

Figure C.2. (left). The ACAPELLA-5K Sample Processor, including the Core Processor (left of photo) and high-throughput Thermal Cycler Module (right of photo)

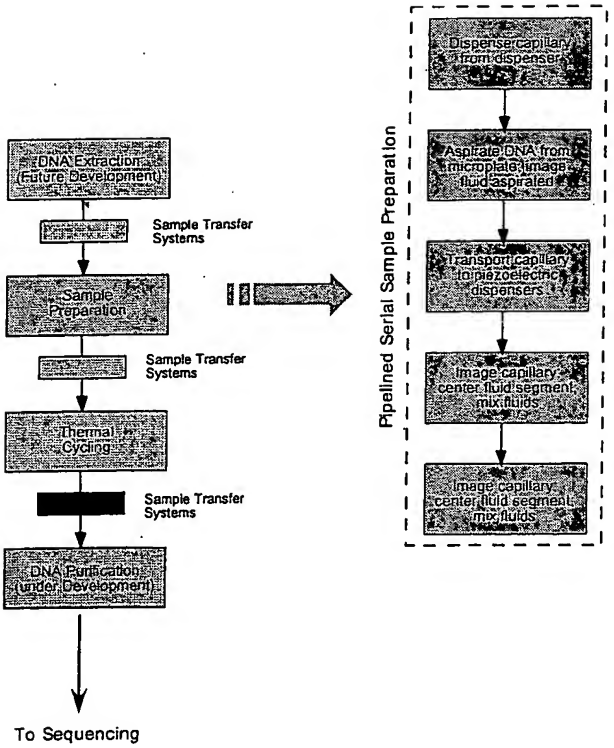

Figure C.3. The ACAPELLA-5K sample processing pipeline for Genome Sequencing applications. The "Sample Preparation" module, which is shown in green and expanded at the right, can be adapted with only modest modification for Protein Crystal-

PIEZO-DISPENSERS

Piezoelectric fluid dispensers manufactured by Engineering Arts, Inc. (www.engineering-arts) are laid out in an arc (left side of Figure C.4.). The piezo-dispensers deliver droplets of 100 pL at a drop-on-demand rate of up to 1 kHz. The rotor positions a capillary in front of each dispenser. Figure C.5 shows a capillary positioned at a fluid dispenser. Figure C.6 is a stroboscopic view of the droplet formation process that is the heart of the Piezo-dispenser. Each dispenser handles a unique reagent. This complement of dispensers allows 8 distinct reagents to be added to the capillaries at will. System Software controls the dispensing of reagents on an individualized, sample-by-sample basis.

MIXING

Mixing is accomplished by oscillating the fluid column back and forth within the capillary at a high rate (3-10 Hz)[51]. Mixing will probably not be used in most protein crystallography setup procedures.

IMAGING OF COLUMNS

The fluid column in the capillary is imaged at several points in the process, in order to determine fluid volume, position in the capillary, and to check for the presence of air gaps [52]. Image recognition software has been developed to analyze these images. Figure C.7 shows a typical capillary image captured directly from one of the on-board cameras.

ACTIVE FEEDBACK TO OBTAIN CONSTANT VOLUME-TO-VOLUME RATIO

The imaging system is used to precisely determine the initial aspirated volume of DNA. Downstream reagent volumes can be adjusted to maintain constant concentration in the final mixture, even in the presence of significant initial volume variations.

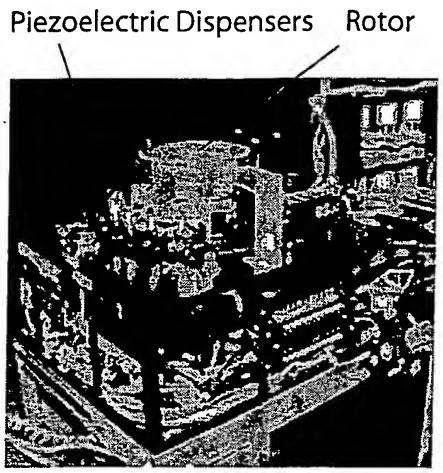

Figure C.4. The ACAPELLA-5K at the center is surrounded by the capillary loader (behind, no visible), dispenser platform (left), and offloader (right).

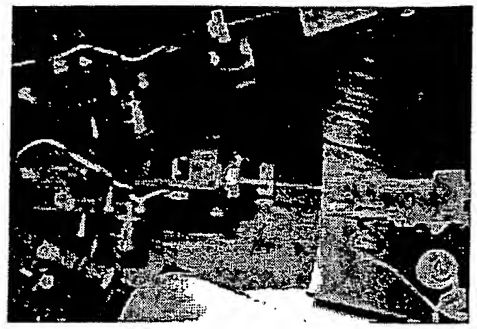

Figure C.5. A chuck (left) with capillary and a Piezo-dispenser (right) are shown in "shooting" position.

Figure C.6. (right). Stroboscopic view of droplet formation at the nozzle of the Piezo-dispenser

THROUGHPUT

The current A5K Core Processor was designed to prepare 5000 samples in an 8-hour time period; this throughput has been successfully demonstrated.

For the Protein Crystallography application, we have set a minimum throughput requirement of 500 samples/hour, with a goal of 1000 samples/hour. We believe that the higher value should not be extremely difficult to attain, since there are several process simplifications (e.g., removal of the aspiration stage) that occur in going from the Sequencing to the Protein Crystallography application.

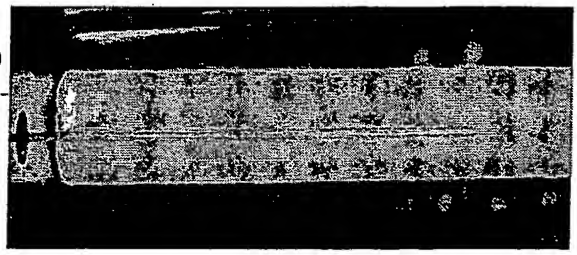

Figure C.7. A captured image of a partially-full capillary. The center background area is an LED illuminator that is specifically designed to provide highly uniform illumination.

C.2 PRELIMINARY CRYSTALLIZATION EXPERIMENTS WITH THE ACAPELLA-5K

To demonstrate the utility of the A5K machine for crystal growth, we have prepared capillaries from several proteins with a number of different precipitants. Precipitants were aspirated from a 96-well microtiter plate and the proteins were shot from one of our piezo-dispensers. The ratio of precipitant to protein solution volume was typically ~ 1:1, and the aspirated and dispensed volumes were ~ 0.3–0.5 µl. Some difficulty was encountered with clogging of the piezo-dispenser by the protein solution if it was allowed to sit without firing for more than a few seconds. This clogging may be due to evaporation of water in the solution exposed at the tip of the dispenser and also to a tendency of the protein to stick to the walls of the very small dispenser aperture (~ 60 µm). In fact, a preliminary experiment to look at the dispenser with a video microscope showed the buildup of strong reflectivity on the tip associated with clogging of the dispenser, suggesting that polymerization was taking place. This problem was avoided as much as possible by keeping the piezo-dispenser firing once started. Further work will be needed to control this problem, and is planned (see Section D4.3)

A list of proteins dispensed is presented in Table C.1. Crystals have been observed in capillaries containing lysozyme (Figure C.8), bovine insulin and hemoglobin, and LM aldolase (Figure C.9).

After demonstrating successful crystal growth in the glass capillaries, we investigated the use of plastic capillaries, which will allow grown crystals to be directly irradiated with X-rays in-place. This is possible because of the much lower X-ray scattering due to the lower average atomic number of the plastic capillary constituents. We tried capillaries made from polycarbonate, with the same outer diameter as our glass capillaries. The in-

Table C.1

Crystallization Preps Run on ACAPELLA-5K

Protein	Conc.	Screen	Capillaries
lysozyme	20 mg/ml	cryo	glass
	40 mg/ml	cryo	glass
bovine insulin	5 mg/ml	wizard	glass
bovine hemoglobin	20 mg/ml	wizard	glass
L' mexicana aldolase	10 mg/ml	cryo	glass
	10 mg/ml	PEG/ion	glass
	10 ml/ml	PEG/ion	plastic
Declymaltoside	0.2%	cryo	glass
LM aldolase + 0.2% Declymaltoside	10 mg/ml	cryo	plastic
Pfalziparum aldolase	6 mg/ml	cryo	plastic

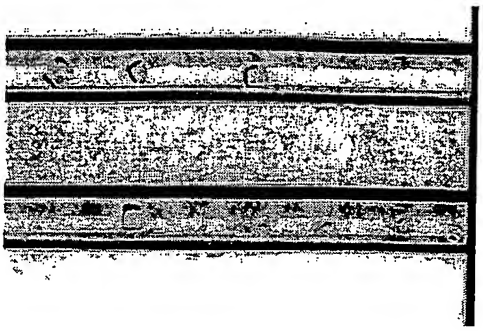

Figure C.8. Lysozyme crystals obtained in glass capillaries prepared with ACAPELLA

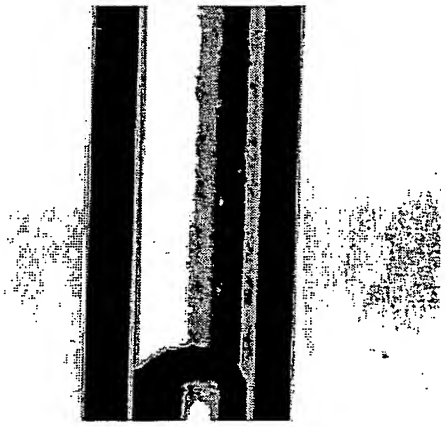

Figure C.9. Leishmania mexicana aldolase crystals grown with ACAPELLA preparation.

side diameter was smaller in order to increase the wall thickness and hence, the stiffness of the plastic capillary. Mechanically, the plastic capillaries worked well without retuning the of the A5K machine. Some difficulties were observed due to jamming of the plastic capillaries in the hopper that loads capillaries into the mixer heads and due to flexing of the capillaries in the cassettes used for offloading and storing the capillaries. These difficulties can be overcome by modifying the A5K to accommodate the characteristics of the plastic capillaries, as described in Section D.2.

The hydrophobic nature of the plastic surface also prevented surface tension from drawing the fluid into the capillary during aspiration of the precipitant and dispensing of the protein. During dispensing, the protein shot from the piezoelectric dispenser would simply ball up on the end of the plastic capillary and prevent further droplets from entering the capillary. We solved this problem by dividing the dispensed protein volume into several bursts of ~ 400 droplets and alternately firing the droplets and sucking the fluid into the capillary with the internal piezoelectric disc in the mixing head. We refer to this process "coordinated dispensing." Adjusting the delay between firing the dispenser and sucking the fluid into the capillary allowed us to fill the capillaries to the desired volume.

C.3 Imaging of crystals in capillaries

Image Acquisition In The Capillary Geometry

We have obtained numerous images of crystals grown in both glass and plastic capillaries. Optimization of the resolution and contrast of these images is a specific goal of the project, and is discussed in further detail in Section D4.4.

Preliminary Algorithm Tests

Our proposed approach for automated crystal detection is based on a class of algorithms that are sensitive to the temporal change of the image due to growth. We have performed a preliminary test of a very simple version of such an algorithm.

As a simple illustration of frame differencing, we have taken an image of a crystal in a capillary. The source image is shown in Figure C.10. The rightmost crystal was extracted from the scene, artificially reduced to 60% and 80% of its size, and then reimposed in the capillary image, as shown in Figure C.11. The difference between these two frames appears in Figure C.12 and represents the growth in the crystal in this "time period."

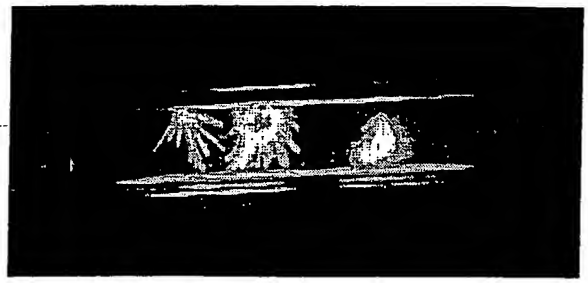

Figure C.10. Initial image used for the simulated image test of differencing algorithms. The crystal at the right was extracted from this image, and used for the test.

This example was taken under ideal conditions: no noise, intrinsically perfect registration, constant illumination, no movement of crystals or fluid boundaries, etc. In reality, registration, normalization for differing illumination conditions, and noise filtering must be part of the full algorithm.

As an illustration of how wavelets can be used to distinguish between different types of crystals, we have compared the high frequency content of two of the crystals shown in Figure C.10 [53–56]. Figure C.13 (left) is the wavelet transform (enhanced) of the crystal on the right, while Figure C.13 (right) is the wavelet transform (enhanced) of the crystal on the left. In both cases, white represents large wavelet coefficients. Further, the white square in the upper left hand corner of each image is the lowest frequency information in the image; as expected, it is large in both images.

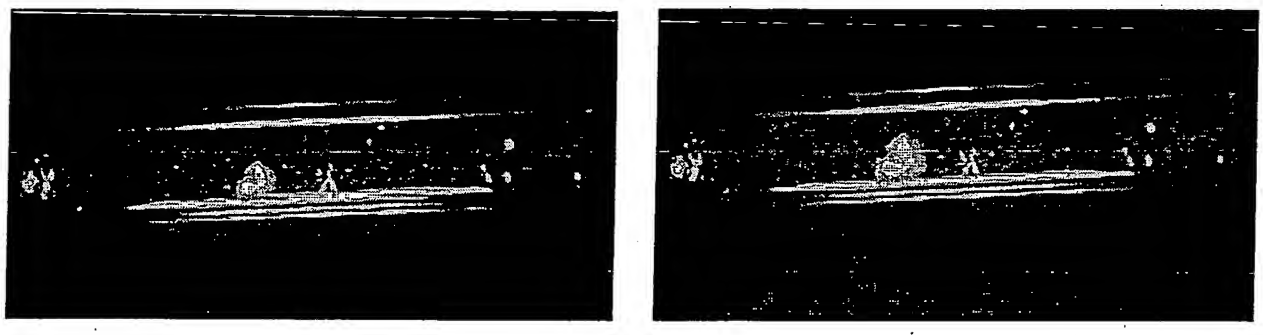

Figure C.11. Simulated crystal growth images, generated by scaling the crystal from Figure C.10 to 60% (left) and 80% (right) of its original size.

Figure C.12.. Difference image showing simulated isolation of crystal "growth". Note the shell-like behavior of the difference image, as the simulated crytal grows away from the capillary wall at the base of the crystal.

These images show that the high frequency wavelet coefficients are significantly higher for the needle-like crystal at the left than for the more monolithic crystal at the right. The high frequency information can be used as a feature in classifying different types of crystals.

C.4 Freezing of crystals in capillaries

We have demonstrated direct freezing of crystalline samples in capillaries. In this case, the surrounding solution had to be replaced with a suitable cryoprotectant solution, a mixture of 0.1M sodium acetate pH4.5, 1M sodium chloride and 40% w/v glycerol. The cryoprotectant was drawn into the capillary by pipette carefully to avoid dislodging the crystals, which were stuck to the sides of the capillary. The solution was expelled and fresh solution drawn up again three times to replace the original buffer with the glycerol solution. The capillary was mounted under the cold nitrogen stream on the X-ray goniometer so that the whole capillary was inside the cold stream. The capillary was held in place with modeling clay. Only the lowest part of the solution close to the modeling clay did not freeze as a clear glass, and this part was well outside of the X-ray beam so there were no strong diffraction rings caused by ice crystals. Crystals frozen by this technique were used to obtain the X-ray diffraction data shown in Section C.6.

An important aim of our Research Project is development of Direct Cryoable Precipitants. This will eliminate the need for the fluid replacement step that was needed for this demonstration.

C.5 X-ray Diffraction Results

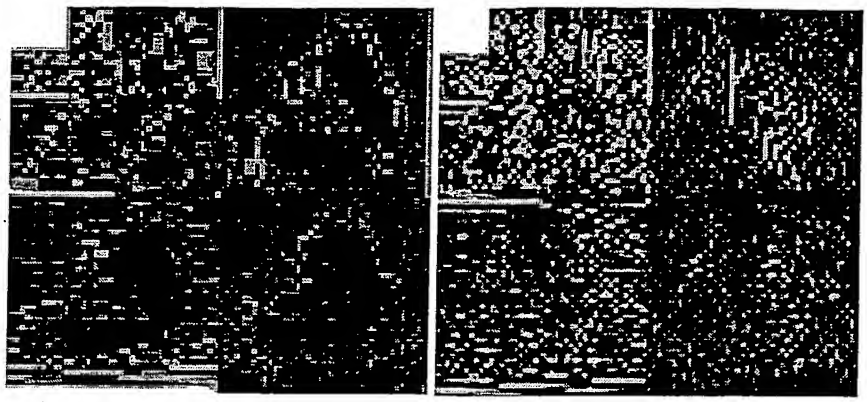

Figure C.13. Wavelet spectra of the needle-like crystal (left spectrum) and monolithic crystal (right spectrum) of Figure C.10.

Lysozyme diffraction data

Crystals were grown in plastic capillaries by mixing equal amounts of 40mg/ml lysozyme in 0.1M sodium acetate pH 4.5 and 2M sodium chloride in the same buffer. These plastic capillaries were the same ones used on ACAPELLA, but the solutions were pipetted by hand. The crystal to detector distance was 150mm and the images collected were of 1 degree oscillations for 15 minutes each. 60 degrees of data were collected and the images were processed and scaled using the Denzo and Scalepack from HKL Research. Figure C.14 shows the result.

Leishmania mexicana Aldolase diffraction data.

Crystals were obtained in glass capillaries using the A5K. The protein concentration was 10mg/ml in a buffer of 0.1M TEA pH8.0 and 150mM sodium chloride. The protein was tested for crystallization against the PEG/ION screening solutions from Hampton Research. The best crystals resulted from the mixture containing 20% PEG3350 and 0.2M dibasic Ammonium citrate using equal amounts of the protein and precipitant.

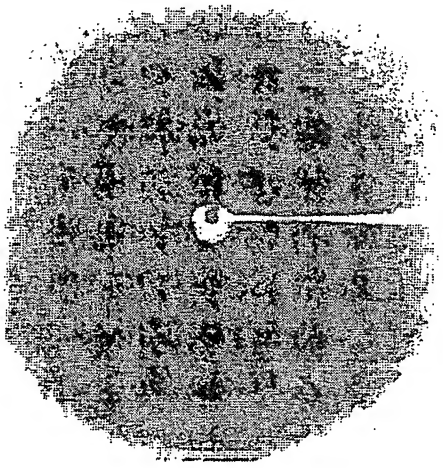

Figure C.14. X-ray diffraction pattern of lysozyme crystal grown in a plastic capillary

Glass capillaries are unsuitable for X-ray diffraction experiments, so the crystals were expelled from the tube. One crystal was transferred to a drop of Paratone-N oil (Hampton Research) using a nylon loop. The remainder of the buffer solution in contact with the crystal was wicked away with a piece of filter paper moistened with the original buffer solution. The crystal was again picked up with a nylon loop and mounted beneath the cold nitrogen stream on the RaxisIIc area detector. X-rays from the Rigaku generator operating at 50KV and 100mA and 1 degree rotation images were collected in 30 minutes with the detector set at a distance of 130mm. Data were processed with Denzo and Scalepack (HKL Research). The crystal had a space group of $P2_12_12_1$ with unit cells of 85.05, 117.91 and 161.60 Angstroms. Final scaling statistics for 106 degrees of rotation data are in Table C.2.

C6. Direct Cryoable Crystallizaton Solutions

The Hol grouop is taking steps to expand the number of "DC" crystallization solutions. This end, cryoprotective agents like glycerol, low-molecular-weight PEGs, cryo-salts, etc. are added in increasing concentrations to existng screens, to see when they become "direct cryoable." The PEG3350/ion screen from Hampton has been made into DC-format in several ways. We are testing to see if this information can be used to obtain DC variants of these poplar screens. In this way, the repertoire of DC precipitants can be significantly expanded.

C7. Co-Crystallants

The Hol group has frequently observed the dramatic effect of additives on crystal growth. Within the context of the SGPP consortium, Drs. Erkang Fan (see letter of support) and Christophe Verlinde are currently engaged in systematic development of additives that are not in themselves traditional crystallizing agents; i.e., they do not act primarily to reduce the solubility of proteins. These new agents, which we call co-crystallants, are of two classes: "freezers", or compounds that have a high probability of stereo-specific binding to proteins, usually in a functionally important site, limiting their inherent flexibility; and "glues", or compounds that have a reasonable probability of being involved in protein-ligand-protein interactions. Both classes should act to promote

nucleation and crystal growth. Our efforts are guided by the observation that certain "privileged" small molecules have a high propensity for binding to proteins [57,58]. The Fan-Verlinde collaboration is designing combinatorial co-crystallant libraries using the binding motifs as components, and will selectively modify the binding motifs to contain one or more (up to four) bromine atoms, for MAD phasing downstream.

Table C.2
Statistics for L' mexicana Aldolase X-ray Diffraction Data

Shell Lower limit	Upper Angstrom	Average I	Average error	Average stat.	Norm. Chi**2	Linear R-fac	Square R-fac
100.00	5.39	2155.8	114.6	89.0	1.028	0.066	0.062
5.39	4.27	2819.8	181.5	154.2	1.100	0.083	0.080
4.27	3.73	2606.3	191.2	159.3	1.239	0.097	0.094
3.73	3.39	1923.0	167.0	145.9	1.293	0.117	0.113
3.39	3.15	1258.4	141.2	132.6	1.078	0.135	0.127
3.15	2.96	811.9	132.6	128.2	0.995	0.186	0.173
2.96	2.82	589.8	132.4	129.9	0.931	0.239	0.223
2.82	2.69	487.9	132.6	130.7	0.875	0.282	0.256
2.69	2.59	386.9	134.9	133.7	0.884	0.359	0.332
2.59	2.50	328.3	136.9	136.0	0.851	0.434	0.388
All reflections		1354.5	146.5	133.7	1.041	0.124	0.101

One strategy being followed is the development of libraries based on substituted guanidiniums, which can attach hydrophobic moieties to the charged central guanidinium scaffold, thereby combining affinity for hydrophobic pockets in proteins with solubility. The Fan group and others [59–61] have developed resin-bound guanidinylation reagents which efficiently synthesize substituted guanidiniums using solid support. .

C8. Combinatorial Libraries

The Hol group has been involved in several collaborations with chemists employing combinatorial chemistry to arrive at inhibitors of drug target proteins, e.g., where thousands of compunds were tested for inhibitory activity against Trypanosomatid GAPDH.

D. Research Design and Methods

D.1 Overview

The proposed Research Project is divided into three phases:

Phase I – Basic Development

We have already shown in many respects, via a series of small demonstrations, that ACAPELLA technology can be adapted to the protein crystallography mission. However, a number of fundamental development tasks remain before it is appropriate to undertake design of a prototype processing system. These tasks will be completed in Phase I. These tasks occupy approximately the first 18 months of the proposed 4-year program.

Phase II–Prototype System Development

The 2nd phase of the project encompasses design, fabrication, and functional testing of a complete prototype system. This phase will require approximately 18 months for completion.

Phase III–Prototype System Testing

With any new processing technology, it is critical that it be tested extensively and intensively in a realistic operating environment. Only realistic testing will uncover the kinds of problems that prevent real users from meeting their goals. Phase III is a one-year period of testing in Dr. Wim G. J. Hol's Biomolecular Structure Center in the Structural Genomics of Pathogenic Protozoa (SGPP) Consortium of the NIH NIGMS.

D.2 Project Team

The proposed project builds on the ACAPELLA development team. Letters of intent from the various participants are attached. This team has proven itself as innovative, efficient, and responsive to the needs of users. The team includes the personnel of the Genomation Laboratory, with Dr. Deirdre Meldrum as Principal Investigator. Dr. Meldrum will be the Principal Investigator for the proposed development project. The team also includes Orca Photonic Systems, Incorporated. Orca is a small company specializing in automation, sensing, and optical

measurement technologies. Orca formally functions as a subcontractor to the Genomation Laboratory. The relationship between the two organizations is highly collaborative, as has been demonstrated by more than 5 years of extremely productive development and engineering work in the various ACAPELLA development programs. The existing team will be augmented by Prof. Eve Riskin (E.E.) and Prof.Richard Ladner (Comp. Sci). They are acknowledged experts in image compression, image analysis, and motion compensation.

For this work, the ACAPELLA team will be joined by the Biomolecular Structure Center in the SGPP Consortium, led by Professor Wim Hol, wholeis also an HMMI investigator. Dr. Hol's laboratory is a world-recognized center for crystallographic structure determination.

D.3. Preliminary System Architecture

Figure D.1 shows a preliminary architecture that has been developed for purposes of this proposal. This architecture has been used to define development tasks and estimate system capabilities. It is presented here to assist the reader in understanding the critical elements of system development. This architecture represents both a hardware schematic, and a development pathway that makes maximum use of the strengths and capabilities of the existing ACAPELLA-5K Core Processor.

Capillary Filling. Capillaries will be filled on a modified version of the ACAPELLA-5K. All fluids, including proteins solutions, will be dispensed with Piezo-dispensers to allow volumes to be delivered at the 50 nL level.

Tape and reel Capillary Transport and Handling. Several methods are under consideration for moving and handling capillaries throughput the system. Single-capillary robotic hand-gripper schemes are useful where processing speed is limited, where processes are variable, and for moving capillaries where switching decisions must be made (for example, between multiple Capillary Growth/Storage modules).

For high-speed, pipelined capillary handling, we are strongly considering the "tape and reel" approach that is widely used in automated electronics assembly. This approach involves taping many components (capillaries) into a long band, and handling them as a continous or quasi-continuous belt. Capillaries will then be removed from the tapes near the end of the pipeline by shearing the ends of the capillaries. These processes are described further in Section D.4.

Figure D.2 shows one existing "low-tech" version of the tape and reel approach. In this case, electronic components (Schottky diodes) are provided by the manufacturer in tape and reel format. The reels of taped

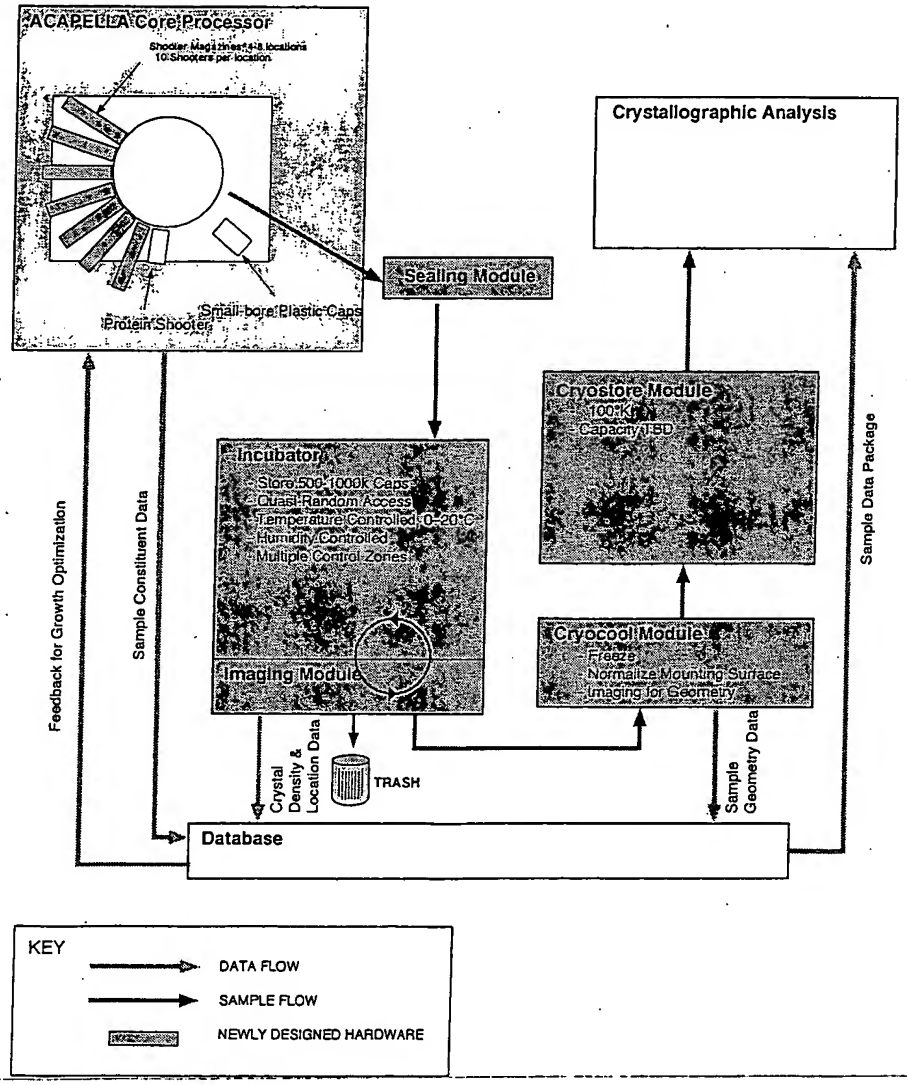

Figure D.1. Preliminary Architecture for the complete Capillary-Based Protein Crystallography Processor.

components are mounted directly on dispensing hubs of automated assembly machines. The tape and reel approach provides a low-cost, high-density, and reliable method for handling of small components.

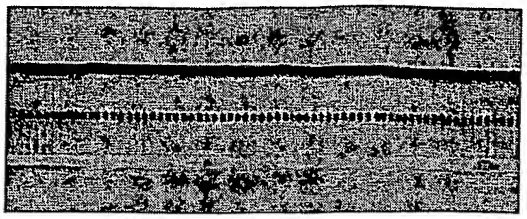

Figure D.2 Example of tape-fed components, in this case high power Schottky diodes used in the ACAPELLA-5K Thermal Cycler Power/Control Modules

There are several options concerning the point at which the capillaries are moved into tape. It is not practical to repeatedly move into and out of the tape format, so once in tape capillaries must stay in tape until the end of the pipeline. These options are:

1. Right from the beginning of the pipeline, including handling on the ACAPELLA-5K.
2. After filling, but before sealing.
3. After sealing, but before storage in the Capillary Growth/Storage Module.

Capillary Sealing. Capillaries will be sealed immediately after filling. Sealing methods under consideration include thermal fusion, ultrasonic welding or polymer plugs, among others.

Capillary Growth/Storage Subsystem. The Capillary Growth/Storage Subsystem will be implemented as multiple modules. The use of multiple modules allows the maintenance of multiple growth environments (temperature and humidity), supports the use of multiple Imaging Stations to achieve the needed imaging throughput, and allows the capacity of the storage system to be easily scaled to different levels for the needs of different users.

Cryofreezing/Storage. After testing for crystal growth, tapes containing groups of capillaries exit the Growth/Storage module. These tapes will move to the Cryofreezing/Storage Module. Individual capillaries of interest are frozen in a gas stream, and then removed from the tapes by cleaving of the capillary ends. This serves to "clean up" the capillary ends in preparation for nounting for crystallography.

X-ray Diffraction Interface. Capillaries from the Cryostore are mounted in reusable precision handlers for diffraction analysis. The mounted capillary will be imaged to determine the location of the crystal relative to reference points on the mount.

Information Management. It is also important to note from Figure D.1 that information flow is no less important that hardware and sample flow. At the point of crystallographic analysis, we have have a package of data containing at least sample identity and high-precision crystal location data.

D.4 Phase I-Basic Development

D4.1 System Architecture for Integrated Capillary-Based Processing for Protein Crystallography

In a hardware development program such as this, many distinct technological and design elements are being developed in parallel. It is crucial to have some kind of working definition of requirements and functions needed at the subsystem level. Without such a baseline, there is a great risk that the exploratory R&D effort will not adequately serve the needs of the ultimate hardware design and realization.

For this purpose, we intend to develop a complete Processor Architecture for the entire processing system. Because it is needed to guide other proposed efforts, it will be completed as early as possible within the project schedule.This architecture study must provide several results.

1. define the processing flow from end to end;
2. define and partition the major functional subsystems of the final system;
3. establish the major quantitative requirements on the performance of the entire system (e.g., functionality and throughput);.
4. define initial quantitative requirements for the subsystems.

D4.2 Optimization of Capillary-Based Crystallization

As discussed in Section C.2, we have successfully demonstrated crystallization of a small range of proteins in both glass and plastic capillaries. To ensure the success of the proposed Automated Processor, we must extend these studies to provide an extensive range of validated protocols that will be supported by the final machine design. Some of the issues that must be resolved by this study are:

1. choice of capillary material (i.e., glass, plastic...), with plastic preferred since it can be used for direct X-ray diffraction studies,
2. acceptable and minimum range of protein sample volumes,
3. evaluation and testing of alternate diffusion regimes (i.e., mixed, liquid-liquid, liquid-gas-liquid,
4. effect of sealing (i.e., sealed, unsealed, porous seal),
5. optimum capillary geometry (i.e., length, bore, inside and outside diamters) to meet both machine and protocol requirements.

These questions will be answered by a series of crystallization tests performed using an existing A5K Core Processor. Such a processor is currently being built for delivery to Dr. Hol's unit of the SGPP Consortium.

D4.3 Piezo-Electric Dispenser Optimization for Protein Solutions

Extensive effort and materials are expended in purifying individual proteins for crystallographic analysis. The typical yield from a protein purification column is on the order of 0.1 mL to 0.5 mL of protein solution with concentrations varying from 5 mg/mL to 40 mg/mL. The fluidic properties of such protein solutions can vary to a significant extent from one solution to the next as a function of molecular weight, concentration, and other additives (e.g., surfactants, buffering agents, and so on). Current manual methods for protein crystallization in capillaries require a volume of 0.5 mL per 1000 crystallant solutions tested while the current state of the art utilizes 0.1 mL per 1000 crystallization mixtures. While this protein volume, 100 nL, begins to press the boundary defining the current state of the art, we propose to further extend our capabilities to 50 nL volumes.

We possess enabling technology for precision dispensing of 33 picoliter droplets at high rate (up to 12.5 kHz) using a piezodispensing technology (Engineering Arts, Mercer Island, WA). We have extensively tested the use of piezo dispensing devices for use with aqueous dilute solutions, alchohol, formamide, dilute and concentrated protein solutions, and the dispensers perform well during normal operations. However, several unique problems arise when using concentrated protein solutions. First, at the tip of the dispensers a "skin" of protein forms on the orifice of the piezo-dispenser if left in an idle state for an extended period of time. Once formed the skin causes the dispensers to cease to function. Second, during extended operation an aggregation of adsorbed protein collects on the dispenser nozzle tip, concentrated around the nozzle orifice. If this skin, and/or the adsorbed protein is not allowed to form and the tip of the dispenser is kept clean then reliable dispenser performance is obtained.

Dispenser optimization methods directly address the mitigation and/or removal of the primary modes of dispenser failure when dispensing proteins. Three complementary approaches are proposed: 1) coating of the dispenser nozzle to mitigate protein adsorbtion, 2) periodic cleansing and capping of the dispenser tip, and 3) periodic dispense of droplets during idle machine cycles. Two primary coating techniques will be evaluated, silanization of all dispenser glass surfaces, and deposition of a plasma polymerized tetraglyme (pp4G). Silanization chemistry is common place and the only innovation required is the development of a custom closed-loop fluidic system to perform the necessary chemistry steps. The plasma deposition of pp4G is a unique capability available at the University of Washington through the laboratory of Prof. Buddy Ratner. Both methods and combinations of the two will be examined for enhanced resistance to protein fouling. Custom cleansing and capping of the dispenser nozzle has been demonstrated by simple manual experiments where a non-fibrous damp swab was gently touched to the nozzle top and ever so slightly agitated in a orbital motion. The process was very effective in the removal of the "skin" phenomena and also effective in removal of accumulated protein residue around the dispenser nozzle. As it is more ideal to never allow a skin to form, periodic dispense during idle machine cycles helps to minimize unrecoverable clogging events.

Another essential component of optimization of dispenser methods is the delivery of small volumes of reagent

reliably to the piezo-dispensers. In general, 100 µl volumes must be accommodated with not a drop (or nanoliter) to spare. To achieve this demanding specification we will develop custom delivery ampules that interface directly with the piezo-dispensers and our automatic load/unload dispenser controls.

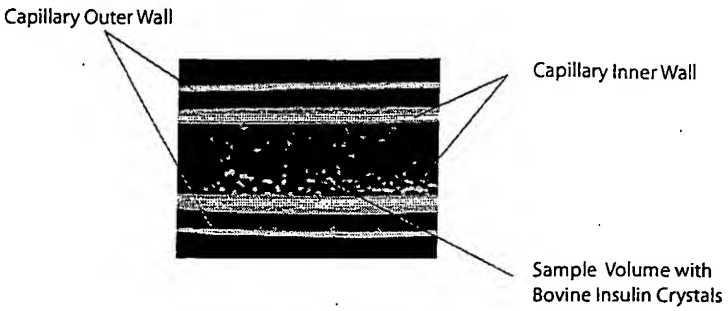

Figure D.3. Captured image of a capillary bore made during our preliminary studies.

D4.4 Imaging Development

To achieve system goals, it will be necessary to acquire and analyze samples images at a sustained rate of order 1/sec. Phase I development of Imaging for Automated Crystal Detection addresses both hardware and methods.

Imaging Optics Design and Test

Images for crystal detection must be acquired directly from the cylindrical capillary, which will most likely be made of plastic because of the direct X-ray diffraction advantage. The cylindrical shape creates significant optical aberrations and imperfections that must be managed. The illumination must be configured to provide maximum contrast for crystals, and minimum visibility of capillary surface imperfections and background artifacts. In addition, optical access to the capillary will be limited by the surrounding transport machinery, which is needed to support the fast detection pipeline (~1/second) mandated by system throughput needs.

Microscopy of crystallites contained in capillaries is hindered by the lens power of the capillaries themselves. Figure D.3 shows an image of bovine insulin obtained in our preliminary studies, which helps to define the problem. The thick wall of the capillary acts as a strong cylindrical lens, and introduces large amounts of astigmastism when using a normal imaging system. Astigmastism causes small structures within the capillary to appear elongated and out of focus in one dimension or the other.

Compensation of the intrinsic capillary astigmatism appears to be possible by introducing offsetting cylindrical lens power at another position in the optical train. As an example, we show here a numerical ray-trace for a relay lens system with a magnification of approximately 5.0, which is roughly appropriate for imaging a capillary onto a CCD. For this example, ideal paraxial lenses were used for simplicity. The optical system layout is shown in Figure D.4, while the capillary optical interface appears as Figure D.5.

A spot diagram (which shows the spot shape of what should ideally be a single point) for the uncorrected case is shown in Figure D.6. In the uncorrected case, rays from a single object point form a strongly elongated spot. Two spots are shown, for a point centered within the capillary, and for a point 50 μm to the side of the axis. Refocusing can reduce the spot length, but only at the cost of increasing its width. Figure D.7 is the spot diagram (at the same scale) with optimum cylindrical power on one surface of the compensating lens. The oval shows the Airy disc dimension—an approximate measure of the diffraction-limited (ideal) spot size.

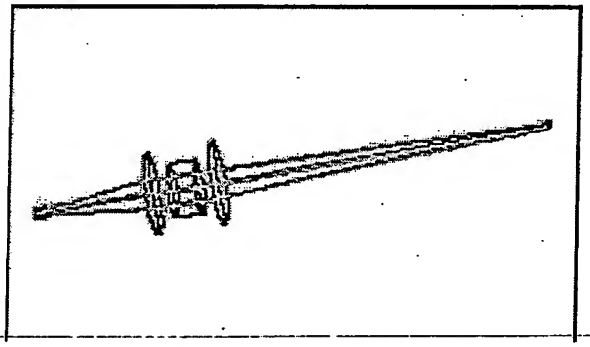

Figure D.4. Simplified optical system used for preliminary analysis of capillary imaging optics with internal cylinder compensation.

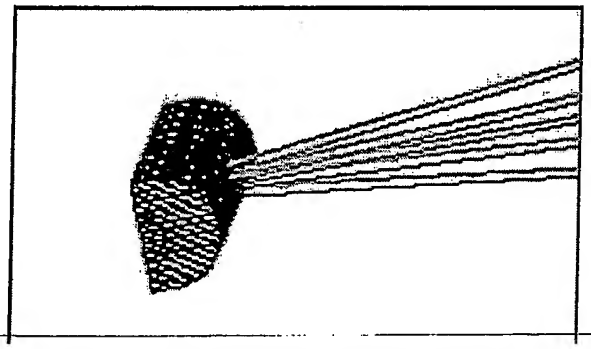

Figure D.5. Ray-trace view of the capillary interface

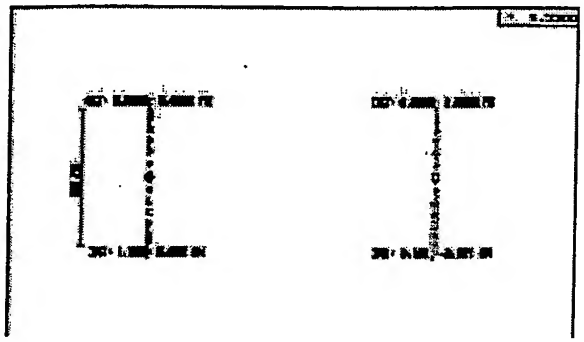

Figure D.6. Spot diagram of the uncorrected capillary imaging system. Note the strong blurring in 1 axis, which is the expression of astigmatism

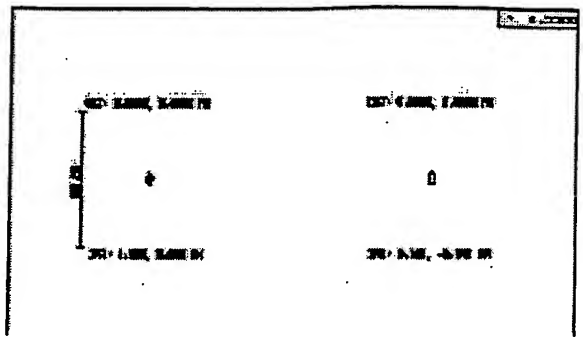

Figure D.7. Spot diagram for the case of an idealized imaging system with cylindrical correction. Note the dramatic reduc-

In a real design, there is a wide range of other issues that must be considered. These include the aberrations of real lenses, and the increasing difficulties of making the correction as higher resolution is desired. While space precludes including the details, Table D.1 shows a tradeoff between resolution and depth-of-field that cannot be avoided. The choice of f-number needed to obtain high resolution implies limited depth of field, and therefore multiple images per capillary. If we design for large depth of field to maximize throughput, then resolution will suffer. Careful management of such tradeoffs is key in the optical system design process. We will complete the detailed design of a viable optical system using this kind of analysis and tools, and build a low-throughput mockup of the entire imaging stage for testing and characterization.

Table D.1

Resolution *vs* Depth-of-Field Tradeoff

f#	Resolution (μm)	Depth of Field (μm)	Exposures to cover 100 μm
10	6.1	120	1
8	4.9	77	2
6	3.7	43	3
4	2.4	19	5+
3	1.8	11	9
2.5	1.5	7.5	13
2.0	1.2	4.8	21
1.5	1.0	2.7	37

Imaging Algorithm Development and Selection

The concept of detecting crystallization by image acquisition and processing is not new. Many investigators have attempted to develop algorithms to implement such a capability, with limited success. To date, most crystal detetion algorithms of which we are aware are based on detection of specific "crystal-like signatures" of one form or another, found within an image at a given point in space and time. We are proposing to use a very different class of algorithms, which exploits the fact that crystal growth is a *change* of the sample state over time.

The motion compensation routines used in most video compression algorithms like the MPEG family of codecs compare an image frame at one time with that at a previous time. They track how objects in the image move from frame to frame, predict an incoming frame, and then code the difference between the incoming frame and its prediction. A similar approach can be used to detect if and where crystal growth has occurred. Because objects are, for the most part, not moving in our situation, a simple difference operation should be sufficient to detect differences (i.e., possible crystal growth) between successive frames. Additional signature analysis will determine whether the difference signal is due to growth or motion. A reference frame is stored immediately after sample preparation. Samples are then periodically re-imaged. The new image undergoes a simple frame-difference operation with the reference image to isolate temporal changes. Such changes could result from crystal growth, noise, or other artifacts such as changes of the fluid boundaries due to evaporation. This approach, to a very high degree, will remove static artifacts, such as imperfections on the capillary exterior, from the analysis.

Once detected, classifying crystals as to their potential diffraction utility is equally challenging. Crystals are

highly varied in appearance. There is no simple image-space definition of a "crystal," which has been the bane of previous imaging implementations. We will use a multiresolution wavelet approach [53,54]. Wavelets intrinsically classify the image over a wide range of spatial frequencies. We will acquire wavelet spectra for a wide variety of actual crystals in capillaries, and test discrimination algorithms against these spectra. We will also investigate the use of wavelet packets for crystal growth detection [55,56]. Wavelet packets have worked well in situations where images have a high degree of high frequency information, such as edges and facet structures. Our expectation is that the low frequency wavelet sub-bands will help to identify "rock-like" crystals, while the higher frequency sub-bands can aid in identifying crytals with structure (needles, ...).

Open questions include the number of wavelet or wavelet packet decompositions and how to distinguish between noise and high frequency signal. We must also implement algorithms to deal with changes in the fluid boundaries due to fluid evaporation or migration, image normalization for differing illumination levels and conditions, and filtering of noise and defects from the source images before differencing. These questions will be answered by testing candidate algorithms against a library of real crystal images, both in capillary format (data acquired within this project) and in droplet format (data available from the SGPP).

D4.5 X-Ray/Synchrotron Interface Development

Once acceptable crystals are grown within the capillary several additional steps are needed in preparation for crystallographic analysis. Two types of Crystallographic Analysis facilities must be accommodated: conventional X-ray sources used, primarily for initial surveys, and syncrotron sources used for final X-ray Diffraction Analysis.

1. The samples must be cryocooled, to fix the state and location of the crystals within the sample holder.
2. The capillary must be conditioned to define a high precision mechanical interface, allowing geometry to be accurately reproduced at the synchroton.
3. Transport and retention fixtures must be developed to provide the necessary precision with reasonable cost and convenience.
4. Data must be provided to the syncrotron in a standardized format to allow accurate location of the crystal within the beam, once the mounted crystal is delivered to the synchrotron.

Figure D.8 shows the process flow needed. Capillaries judged worthy of crystallography are delivered from the incubator subsystem in tape and reel format, and then removed from the tape by shearing. This serves several purposes. First, shearing allows the central part of the capillary to be removed from the tape and retained. Second, the length of the capillary must be shortened for compatability with existing synchrotron goniometer geometries. Third, shearing removes the ends of the capillary that may be distorted by sealing. The capillary is cryocooled in a Nitrogen gas stream, and finally delivered to a storage pocket. Single-capillary handling is viable at this stage of the process, since the quantity of capillaries to be processed is much smaller than in the incubator.

After storage, capillaries are inserted into a mounting block designed for direct interface to the crystallography facility. A mount that is mechanically compatible with existing synchrotron crystal carriers is contemplated. Figure D.9 shows a schematic view of a preliminary concept for the carrier block. It is retained in the goniometer via a magnetic base. The base interface is keyed, so that the rotational position relative to the goniometer accurately predictable.

Knowledge of the crystal position within the mounted sample is essential for synchrotron-based X-ray diffraction analysis. The relatively small photon beam must be accurately placed on the sample in order to maximize diffraction effiency. After mounting, the crystal will located within the mounted sample by an additional imaging step, and a package of geometry data generated for the X-ray facility.

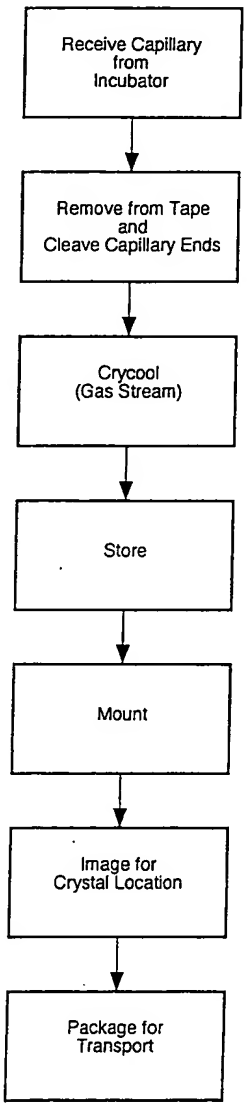

Figure D.8 Process flow for the crytallography interface.

capacity for these modular feature additions.

In the phase I development of the capillary sealing subsystem we will:

1. develop detailed sealing module layouts for both rotary and tape and reel capillary-based core processors,
2. develop sealing module functional specifications for both rotary and tape and reel core processors,
3. design, fabricate, and test a testbed sealing module for both architectures,
4. dvaluate and formulate recommendations for phase II development tasks.

D4.9 Development of 1000 Capillary "Tape and Reel" Capillary Incubator and Imaging Testbed.

Crystal growth requires that capillaries be incubated for a period of time extending up to 3 months, that nucleation and growth of individual crystals be monitored and recorded throughout this interval, and that individual capillaries be extractable from the incubator when crystal geometries are deemed to be successes or failures. Capillaries of relevance removed from the incubation module are then cooled and stored at cryogenic temperatures for subsequent crystallographic analysis. These requirements represent a sophisticated integration of environmental control, imaging and image processing, database management, and hard automation robotics for manipulation of capillaries. Further, due to the lengthy incubation period during which a majority of the samples must be maintained, efficient use of volume within the incubation module, while maintaining precise and uniform conditions, is also required. Eventual capacity of a full scale incubation module cluster will require capacity for up to 1 million capillaries in crystallization survey sets of 1000 each. The primary focus of phase I is the identification, classification, and test of solutions to integrated incubator system requirements. In phase I we will resolve the hardware automation, imaging, and space optimization issues. We will develop a 1000 unit incubation module with a an eye on future scalability.

In the phase I developmental evaluation of incubation module options we will:

1. develop detailed architectural layouts for alternate incubator requirement integration configurations;
2. develop functional specification documents for feasible architectures;
3. design, fabricate, and test architecture subsystem components for reliable long term operation;
4. design, fabricate, and test integrated incubator testbed comprised of proven functional components;
5. evaluate and formulate recommendations for phase II development tasks.

D4.10 Crycompatible Precipitant Development

An important aspect of the propposal is the development of an increasing number of cryocompatible direct cryoable) precipitants. We will continue developing and testing new combinations of organics, PEGS, low molecular weight organic cryoprotectants, s well as a number of cryo-soltas, to have a large set of CC/DC solutons available for testing. We also expect that the literature will continue to provide new DC/CC precipitants, since several relevant reports have appeared very recently.

D5. Phase II Tasks-Development of Complete Prototype Protein Crystallography System

D5.1 Final System Architecture and Requiirements

Before entering into detailed system design, we will revisit the system requirements and architecture. Experience gained in the development of ACAPELLA shows that the requirements perceived by users, and our understanding of how to best meet those requirements, evolve over time.To respond to this reality, the architecture described in Section D.3 will be periodically revisited during the life of the project, and specifically just before detailed design of a prototype system begins. The task is specifically designed to:

- incorporate the results of Phase I,
- maximize performance and minimize cost by incorporating recent developments,
- respond to changing user needs,
- reduce technical risk by adjusting performance goals to realistic state of the technology.

D5.2 Detailed System Design

Detailed system engineering and design of a hardware system such as the one being proposed contrasts very strongly with the usual research project. Based on our current understanding of the design, this process will ultimately involve production of a set of something like 1000 engineering drawings and related documents. Several challenges are inherent in this kind of design:

- The very large number of component designs being developed must fit and work together. This requires a process of intensive internal and external design checking and review.
- Designs must be supported by analyses or experimental results in areas where there are significant uncertainties, or where components are being pressed to their fundamental limits.
- Reasonable design margins must be incorporated, to ensure that the system will meet requirements, even in an imperfect world.
- Designs must be practical to manufacture and support. A prodigious design that cannot be realized in practice is of questionable value.

The project team proposed for this project has extensive experience with these design issues, all of which have been faced and surmounted in the ACAPELLA development projects, and in other major hardware development programs completed at Orca Photonic Systems, Inc.

D5.3 Subsystem Development

As was shown Figure D.1, the proposed system can be naturally and logically segregated into a handful of major systems. This section outlines the steps that will be taken in the development of each major subsystem.

ACAPELLA Core Processor

The ACAPELLA Core Processor will see only minor modifications from the current Genome Sequencing version. The specific changes expected are:

1. removal of the input microplate stage, which will not be useful for the crystallography,
2. incorporation of a Piezoelectric Dispenser design optimized for proteins,
3. adjustment to the design to accommodate plastic capillaries, and
4. modification of the design to accommodate multiple dispenser hotels for both co-crystallant and small-molecule reagent libraries.

These modifications should be straightforward, given successful completion of the development and verification work planned for Phase I. Initial implementation will be of a configuration including a 100-reagent co-crystallant library Piezo-dispenser hotel capability.

Sealing Module

The method for capillary sealing will be resolved in Phase I. Once the fundamentals are proven, design and integration is expected to be a straightforward robotic implementation task, based on a protocol that will be fully-proved in the lab in advance of the final design.

Crystal Growth Unit ("Incubator")

The crystal growth unit must serve a number of demanding requirements. The required capacity and throughput is very high, and reasonable size and cost are also certainly implicit requirements. In order to allow for a typical 3–12 week growth trial time for samples, the capacity of the Incubator must be of order $0.5–2x10^6$ samples, depending on precise usage pattern. The volume of this store can easily become burdensome. If the volume occupied per sample were only 1500 mm^3 (corresponding to packing on 5 mm centers), then just the volume occupied by the capillaries will amount to 1.5 m^3 per million capillaries. Given access and environmental control requirements, it will be very challenging to keep this volume within reasonable limits. Fortunately, there is a large base of appropriate technology available. As described in Section D4.7 and D4.9, we will test the tape and reel technology in the capillary environment well before starting detailed design. For reasons of project cost, we plan to implement a subscale version of the Incubator, in the form of a single module with a total capacity of about 100,000 samples. This will be sufficient to demonstrate the utility of the incubator in an actual production crystallography environment.

Imaging Station

All of the functionality and fundamental algorithms of the Imaging Station will have been extensively tested and optimized in Phase I before reaching detailed design. The remaining challenge will be: a) development of a design that is compatible with in-line operation in the Crystal Growth Unit, and b) implementation of high-throughput image processing hardware that can provide image analysis at a rate of 1 image/second. The first requirement will be met by engineering the Imaging Station as an integral part of the Capillary Store. The latter requirement appears to be well within the capability of modern DSPs (Digital Signal Processors). There will be multiple Imaging Stations, since there will be one or more per Capillary Storage Module. Given the relatively low cost of DSP throughput (~$0.1 per MegaFLOP), and the high degree of parallelization that is practical for most image processing algorithms, it is logical to simply use many independent processors to handle this task.

Cryocool/Storage Unit

Like the Sealing Module, the Cryocool/Storage unit is expected to be a relatively straightforward engineering development task. Before the sample stream reaches the Cryostore Module, the throughput is reduced by a factor of 10-100, since only successful samples are taken to this point. The process can be handled with "robotic hand" approaches operating serially on a single sample.

X-ray/Synchrotron Interface

The Crystallographic Analysis Interface will receive significant development in Phase I (see Section D4.6). By the time we reach Phase II, tested designs will be in hand. Final development will consist of scaling up the design to full throughput, and fleshing out a fully documented engineering design package. Part of the Crystallographic Anaysis Interface is a distinct Imaging Station, used to determine crystal/sample geometry before Crystallographic Analysis. This station will use the same algorithms and imaging configuration as the Imaging Station used for detection of crystal growth. However, the throughput requirements are much smaller, and it is probable that parallel image processing hardware will not be required.

Automated Optimization via Feedback

The proposed Integrated Crystallization System can provide a full screen, including evaluation of results, with minimal human intervention. The next logical step is to feedback these results into new, narrowed and optimized screening conditions. We intend to develop an initial algorithm and software for this purpose, which will be extensively tested in Phase III.

D.6 Phase III Tasks-System Testing

D6.1 Testing of Prototype Integrated Crystallography System

The fourth year of the proposal will be devoted mainly to prototype testing for structural genomics and medicinal macromolecular crystallography. This will be carried out in the context of the regular scientific research of the Hol group, which is devoted to crystal structure determinations of proteins from tropical parasites and the structure-guided development of potential new therapeutic compounds. Numerous proteins are currently under investigation, many of these members of large multi-protein complexes involved in crucial processes in parasites that are the causative agents of malaria, sleeping sickness, leishmaniasis, Chagas' disease and cholera. Recently started projects aim at unraveling multi-protein systems such as:

- the biogenesis of glycosomes in Trypanosomatids which is carried out by a family of 23 or more different peroxins. These proteins interact with each other and the cargo proteins to be imported into the glycosome in many different ways
- the editing complex in Trypanosomatids which consists of a dozen or more proteins which modify and expand the immature mRNA for several proteins by using the sequence information in dozens if not hundreds of small guide-RNAs
- the secretion system by which cholera toxin is translocated in folded form across the outer membrane of Vibrio cholerae, a system involving approximately 16 proteins
- the machinery by which the malaria parasite enters host cells

In addition to these projects it is anticipated that the SGPP consortium will be continued in a way which will

functional, chemical and structural genomics of various pathogens, including tropical protozoa. This will allow the Hol group access to funds to purchase small compound libraries. We expect the Fan group to continue to develop combinatorial libraries for candidate drug discovery that can be incorporated into the testing phase. Dr Fan and Dr Verlinde are currently writing a grant proposal which will, if awarded, result in several combinatorial libraries for inhibiting the iterations between the peroxins and thereby prevent glycosome biogenisis in Trypasonomtiods We anticipate moreover that other chemists will be collaborating in several way with the Hol group. A detailed test plan will be created in year 03.

D.7 Project Schedule

Figure D.11 shows the schedule for the entire project in Gantt chart form.

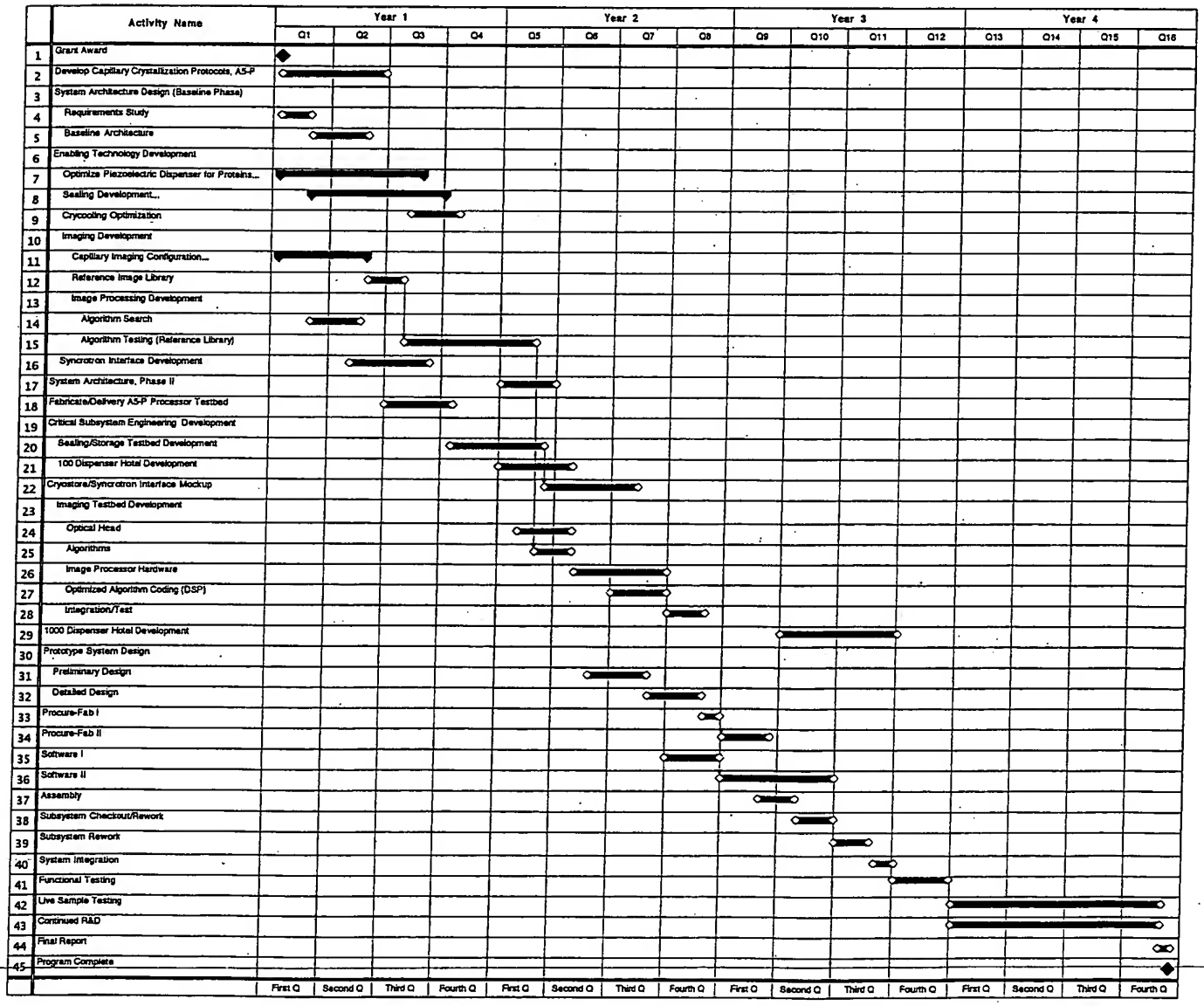

Figure D11. Complete project task breakdown and schedule

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